

IN THE SPECIFICATION

Please amend paragraphs [0213], [0216], [0237], [0238] and [0242] as indicated below.

[0213] Numerous methods and devices are well known to the skilled artisan for the detection and analysis of the markers of the instant invention. With regard to polypeptides or proteins in patient test samples, immunoassay devices and methods are often used. *See, e.g.*, U.S. Patents 6,143,576; 6,113,855; 6,019,944; 5,985,579; 5,947,124; 5,939,272; 5,922,615; 5,885,527; 5,851,776; 5,824,799; 5,679,526; 5,525,524; and 5,480,792, each of which is hereby incorporated by reference in its entirety, including all tables, figures and claims. These devices and methods can utilize labeled molecules in various sandwich, competitive, or non-competitive assay formats, to generate a signal that is related to the presence or amount of an analyte of interest. Additionally, certain methods and devices, such as biosensors and optical immunoassays, may be employed to determine the presence or amount of analytes without the need for a labeled molecule. *See, e.g.*, U.S. Patents 5,631,171; and 5,955,377, each of which is hereby incorporated by reference in its entirety, including all tables, figures and claims. One skilled in the art also recognizes that robotic instrumentation including but not limited to Beckman ~~Aceess~~ ACCESS®, Abbott ~~AxSym~~ AXSYM®, Roche ~~EleeSys~~ ELECSYS®, Dade Behring ~~Stratus~~ STRATUS® systems are among the immunoassay analyzers that are capable of performing the immunoassays taught herein.

[0216] The analysis of a plurality of markers may be carried out separately or simultaneously with one test sample. For separate or sequential assay of markers, suitable apparatuses include clinical laboratory analyzers such as the ~~EleeSys~~ ELECSYS® (Roche), the ~~AxSym~~ AXSYM® (Abbott), the ~~Aceess~~ ACCESS® (Beckman), the ADVIA® CENTAUR® (Bayer) immunoassay systems, the NICHOLS ADVANTAGE® (Nichols Institute) immunoassay system, etc. Preferred apparatuses or protein chips perform simultaneous assays of a plurality of markers on a single surface. Particularly useful physical formats comprise surfaces having a plurality of discrete, addressable locations for the detection of a plurality of different analytes. Such formats include protein microarrays, or "protein chips" (*see, e.g.*, Ng and Ilag, *J. Cell Mol. Med.* 6: 329-340 (2002)) and certain capillary devices (*see, e.g.*, U.S. Patent No. 6,019,944). In these embodiments, each discrete surface location may comprise antibodies to immobilize one or

more analyte(s) (e.g., a marker) for detection at each location. Surfaces may alternatively comprise one or more discrete particles (e.g., microparticles or nanoparticles) immobilized at discrete locations of a surface, where the microparticles comprise antibodies to immobilize one analyte (e.g., a marker) for detection.

[0237] Assays for MMP-9 were performed using murine anti-MMP-9 antibodies generated by Biosite Incorporated using phage display and recombinant protein expression as described previously (U.S. Patent No. 6,057,098). Commercially available MMP-9 antigen was used for assay standardization (Calbiochem-Novabiochem Corporation, San Diego, CA). The immunogen used for antibody production was prepared by Biosite Incorporated. PCR primers were made corresponding to sequence at the 5'-end of human MMP-9 and the coding sequence at the 3'-end of human MMP-9 (Genbank accession number J05070), including six histidine codons inserted between the end of the coding sequence and the stop codon to assist in purification of the recombinant protein by metal-chelate affinity chromatography, primers A (5'(AGGTGTCGTAAGCTTGAATTCAGACACCTCTGCCGCCACCATGAG) SEQ ID NO:1) and B (5'(GGGCTGGCTTACCTGCGGCCTTAGTGATGGTGATGGTGATGGTCCTCAGGGCACTGCAGGATG) SEQ ID NO:2), respectively. The 5' primer also contains 21 base pairs of pEAK12 vector sequence (Edge BioSystems, Gaithersburg, MD) at its 5'-end corresponding to the *EcoRI* site and sequence immediately upstream. The 3' primer contains an additional 20 base-pairs of vector sequence, including 6 bases of the *NotI* site and the sequence immediately downstream, at its 5' end. The vector sequence at the 5'-ends of these primers will form, upon treatment with T4 DNA polymerase, single-stranded overhangs that are specific and complementary to those on the pEAK12 vector. The PCR amplification of the MMP-9 gene insert was done on a 2x 100 µl reaction scale containing 100 pmol of 5' primer (A), 100 pmol of 3' primer (B), 2.5 units of Expand polymerase, 10 µl 2 mM dNTPs, 10 µl 10x Expand reaction buffer, 1 µl of ~~Clontech~~ CLONTECH® Quick-clone human spleen cDNA (Clontech Laboratories, Palo Alto, CA) as template, and water to 100 µl. The reaction was carried out in a Perkin-Elmer thermal cycler as described in Example 18 (U.S. Patent 6,057,098). The PCR products were precipitated and fractionated by agarose gel electrophoresis and full-length products excised from the gel, purified, and resuspended in water (Example 17, U.S. Patent 6,057,098). The pEAK12 vector was prepared to receive insert by digestion with *NotI* and *EcoRI*

(New England BioLabs, Beverly, MA). The insert and *EcoRI/NotI* digested pEAK12 vector were prepared for T4 exonuclease digestion by adding 1.0 µl of 10x Buffer A to 1.0µg of DNA and bringing the final volume to 9µl with water. The samples were digested for 4 minutes at 30°C with 1µl (1U/µl) of T4 DNA polymerase. The T4 DNA polymerase was heat inactivated by incubation at 70°C for 10 minutes. The samples were cooled, briefly centrifuged, and 45 ng of the digested insert added to 100 ng of digested pEAK12 vector in a fresh microfuge tube. After the addition of 1.0 µl of 10x annealing buffer, the volume was brought to 10 µl with water. The mixture was heated to 70°C for 2 minutes and cooled over 20 minutes to room temperature, allowing the insert and vector to anneal. The annealed DNA was diluted one to four with distilled water and electroporated (Example 8, U.S. Patent 6,057,098) into 30 µl of electrocompetent *E. coli* strain, DH10B (Invitrogen, Carlsbad, CA). The transformed cells were diluted to 1.0ml with 2xYT broth and 10 µl, 100 µl, 300 µl plated on LB agar plates supplemented with ampicillin (75µg/ml) and grown overnight at 37°C. Colonies were picked and grown overnight in 2xYT (75µg/ml ampicillin at 37°C. The following day glycerol freezer stocks were made for long term storage at -80°C. The sequence of these clones (MMP9peak12) was verified at MacConnell Research (San Diego, CA) by the dideoxy chain termination method using a Sequatherm sequencing kit (Epicenter Technologies, Madison, WI), oligonucleotide primers C 5'(TTCTCAAGCCTCAGACAGTG) SEQ ID NO:3) and D (5'(CCTGGATGCAGGCTACTCTAG) SEQ ID NO:4) that bind on the 5' and 3' side of the insert in the pEAK12 vector, respectively, and a LI-COR® 4000L automated sequencer (LI-COR, Lincoln, NE). Plasmid suitable for transfection and the subsequent expression and purification of human MMP-9 was prepared from clone MMP9peak12.2 using an ~~EndoFree~~ ENDOFREE® Plasmid Mega Kit as per manufacturer's recommendations (Qiagen, Valencia, CA). HEK 293 ("Peak") cells were expanded into a T-75 flask from a 1ml frozen vial stock (5x10⁶ cells/ml) in IS 293 medium (Irvine Scientific, Santa Ana, CA) with 5% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS), 20 units/ml Heparin, 0.1% Pluronic F-68 (JRH Biosciences, Lenexa, KS), and 50 µg/ml Gentamicin (Sigma, St. Louis, MO). After incubating at 37°C, 85% humidity, and 5% CO₂ for 2-3 days, the cells were expanded into a T-175 flask while reducing the FBS to 2% in the medium. The cells were then continuously expanded 1:2 over a period of 2-3 weeks, establishing a consistent mono-layer of attached cells. Peak cells grown with the above method were

centrifuged at 1000 rpm for 6 minutes, and the supernatant was discarded. After counting the cells to establish the density and checking for at least 90% viability with a standard dye test, the cells were resuspended at 5×10^5 cells/ml in 400ml IS 293 with 2% FBS and 50 μ g/ml Gentamicin and added to a 1 L spinner flask. Then, to a conical tube 5ml IS 293 and 320 μ g MMP-9 DNA were added per 400ml spinner flask. This was mixed and incubated at room temperature for 2 minutes. 400 μ l ~~X-tremeGENE~~ X-TREMEGENE™ RO-1539 transfection reagent (Roche Diagnostics, Indianapolis, IN) per spinner was added to the tube that was then mixed and incubated at room temperature for 20 minutes. The mixture was added to the spinner flask, and incubated at 37°C, 85% humidity, and 5% CO₂ for 4 days at 100 rpm. The cell broth from the above spinner flask was spun down at 3500 rpm for 20 minutes, and the supernatant was saved for purification of the MMP-9. A column containing 20ml Chelating Fast Flow resin (Amersham Pharmacia Biotech, Piscataway, NJ) charged with NiCl₂ was equilibrated with BBS. Then the supernatant from the spinner flask was loaded on the column, washed with BBS + 10mM imidazole, and eluted with 200mM imidazole. The elution was used for the load of the next purification step after adding CaCl₂ to 10mM. A column with 5ml gelatin ~~sepharose~~ SEPHAROSE® 4B resin (Amersham Pharmacia Biotech, Piscataway, NJ) was equilibrated with BBS + 10mM CaCl₂. After loading the antigen, the column was washed with equilibration buffer, and the MMP-9 was eluted using equilibration buffer + 2% dimethyl sulfoxide (DMSO). Polyoxyethyleneglycol dodecyl ether (BRIJ-35) (0.005%) and EDTA (10mM) were added to the elution, which was then dialyzed into the final buffer (50mM Tris, 400mM NaCl, 10mM CaCl₂, 0.01% NaN₃, pH 7.5, 0.005% BRIJ-35, 10mM EDTA). Finally, the protein was concentrated to approximately 0.25 mg/ml for storage at 4°C. Zymogram gels were used to check for production and purification of MMP-9. Western blots were also used to check for activity of the protein. MMP-9 (Oncogene Research Products, Cambridge, MA) was used for comparison of the purified antigen made using the PEAK cell system to known standards.

[0238] Assays for TAT complex were performed using a commercially available murine anti-human TAT complex-specific monoclonal antibody, clone EST1, (American Diagnostica Inc., Greenwich, CT) and murine anti-human TAT complex antibodies produced by Biosite Incorporated using phage display and recombinant protein expression as described previously (U.S. Patent No. 6,057,098). Human TAT complex used for immunization and standardization

of the assay was prepared by incubating human antithrombin III with human thrombin (Haematologic Technologies Inc., Essex Junction, VT) in borate-buffered saline for 15 minutes at room temperature. TAT complex was purified by gel filtration using a 1.5 cm × 100 cm SUPERDEX® 75 resin (Pharmacia, Piscataway, NJ) column that was equilibrated with borate-buffered saline at a flow rate of 1 ml/minute.

[0242] Immunoassays were performed on a TECAN® Genesis RSP 200/8 Workstation. Biotinylated antibodies were pipetted into microtiter plate wells previously coated with avidin and incubated for 60 min. The solution containing unbound antibody was removed, and the cells were washed with a wash buffer, consisting of 20 mM borate (pH 7.42) containing 150 mM NaCl, 0.1% sodium azide, and 0.02% ~~Tween~~ TWEEN®-20 surface active agent (ICI Americas). The plasma samples (10 µL) were pipetted into the microtiter plate wells, and incubated for 60 min. The sample was then removed and the wells were washed with a wash buffer. The antibody-alkaline phosphatase conjugate was then added to the wells and incubated for an additional 60 min, after which time, the antibody conjugate was removed and the wells were washed with a wash buffer. A substrate, (AttoPhos®, Promega, Madison, WI) was added to the wells, and the rate of formation of the fluorescent product was related to the concentration of the marker in the patient samples.